

Direct Effects of Minoxidil on Epidermal Cells in Culture*

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Minoxidil, a potent antihypertensive agent, induces generalized hypertrichosis when administered systemically, or localized hair regrowth when applied topically to sites of severe alopecia areata. The pharmacologic mechanisms by which minoxidil stimulates hair growth are unknown. This study was designed to examine whether minoxidil has direct effects on neonatal murine epidermal cells in culture. In the presence of minoxidil, cultures showed a marked dose-dependent second peak of DNA synthesis 8–10 days after culture initiation. In addition, two morphologically distinct cell types appeared. Indirect immunofluorescence staining with keratin-specific antibody revealed cytoplasmic keratin fibers, suggesting the epidermal origin of these cells. Our experiments demonstrate that minoxidil can affect epidermal cells in culture by altering their growth pattern and phenotypic appearance.

Minoxidil, a potent oral antihypertensive agent that promotes vasodilation also affects hair growth. Patients receiving minoxidil systemically for periods of over 1 month to control severe hypertension were found to have generalized reversible, hypertrichosis [1,2]. This unexpected finding suggested that minoxidil might be useful in the treatment of alopecia areata. In recent clinical studies, topical application of 1% minoxidil to sites of severe hair loss resulted in the growth of terminal hair in approximately half of the treated cases [3]. In investigating the pharmacologic mechanisms through which minoxidil affects hair growth, we utilized a well-characterized neonatal mouse epidermal cell culture system to specifically determine whether minoxidil has direct effects on epidermal cells in culture.

MATERIALS AND METHODS

Epidermal Cell Culture

Neonatal BALB/c mouse skin was cultured by modification of previously described methods [4,5]. Briefly, skin was incubated in 0.25% trypsin (Sigma Chemical Co., St. Louis, Missouri) in phosphate-buffered saline (PBS), pH 7.3, for 45 min at 37°C. After separation of epidermis from dermal components, the epidermal cells were released into complete media (RPMI 1640, 13% fetal calf serum (FCS), 10,000 U/ml penicillin, 10,000 µg/ml streptomycin) and centrifuged through a discontinuous Ficoll gradient for 30 min at 200 g at 4°C. Cells pelleting

in the densest fraction were plated in complete medium (10^6 cells/ml) on plastic tissue culture dishes (Corning, Cambridge, Massachusetts) and incubated at 33°C in a humid, 95% air, 5% CO₂ atmosphere.

Eighteen hours after initiation, treated cultures were re-fed with complete media containing minoxidil (10 µg/ml unless otherwise specified). Media for control cultures contained solvent (propylene glycol:ethanol:water, 50:30:20 v/v) final concentration 1%, or no additions.

Duplicate cultures were pulsed daily with [³H]dThd (1.25 µCi/ml; sp act 20 mCi/mmol) for 4 h. DNA synthesis was measured by incorporation of [³H]dThd into acid-precipitable DNA. Protein was measured as described by Lowry et al [6] and total DNA content was measured according to Burton [7]. Each experiment was repeated at least 3 times.

For subcultures, cells were dispersed by trypsinization (incubation in 0.1% trypsin/0.33% EDTA in PBS for 10 min at 37°C), washed once in PBS and centrifuged at 400 g for 5 min at 4°C. Cell viabilities (assayed by trypan blue exclusion) were determined and 1×10^6 viable cells were plated in complete medium (minoxidil-free) and incubated as described above.

Dermal Fibroblast Culture

Trypsin-separated dermis (derived from neonatal BALB/c mouse skin as above) was minced and incubated in enzyme solution composed of 9:1 ratio of 0.4% collagenase, CLS II, (Worthington Chemical Corp., Freehold, New Jersey) and 0.25% trypsin in PBS, for 60 min at 37°C. The resulting cell suspension was filtered through nylon gauze, washed once in complete medium (RPMI 1640 as above) and centrifuged at 400 g for 5 min at 4°C. Viable cells (5×10^5)/ml were plated onto tissue culture dishes and incubated at 33°C in a 5% CO₂ humid atmosphere. Cultures were fed every 2nd day and passaged twice weekly.

Detection of Keratin by Indirect Immunofluorescence Microscopy

Indirect immunofluorescence microscopy, to verify the epidermal origin of cultured cells, was performed by standard methodology [8]. Briefly, cultured cells grown on glass coverslips were washed twice with PBS, fixed with cold ethanol, and incubated with rabbit antikeratin antibody (kindly provided by Dr. T.-T. Sun, New York University Medical Center) for 30 min at 37°C. After 3 washes, the cells were incubated in the dark with fluorescein isothiocyanate labeled goat antirabbit IgG (Cappel Laboratories, Cochranville, Pennsylvania) for an additional 30 min at 37°C. The cells were washed 3 times and were examined with a Zeiss fluorescence microscope equipped with epillumination. Controls, including incubation with preimmune serum or omission of primary antiserum, were performed in all experiments. Antibodies were titrated so as to give maximum specific binding. All dilutions and washes were made in PBS containing 0.1% sodium azide.

Histologic and Histochemical Staining Procedures

Identification of fibroblasts by histochemical demonstration of leucine aminopeptidase activity as previously described [4,9] was performed on both epidermal and fibroblast cultures.

The presence of cornified envelopes in epidermal cell cultures was assayed by the method of Sun and Green [10].

The oil red O stain for lipid [11] and Kreyberg [12] and rhodanile blue [13] stains to detect keratin were carried out on formalin-fixed epidermal cultures.

RESULTS

Primary Epidermal Cell Cultures

The addition of minoxidil to neonatal murine epidermal cell cultures led to concentration-dependent alterations in their proliferation (Fig 1).

Concentrations of minoxidil of 5–10 µg/ml gave the most

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Abbreviations:

PBS: phosphate-buffered saline
[³H]dThd: tritiated thymidine

marked results, 20 $\mu\text{g}/\text{ml}$ inhibited proliferation, and 40 $\mu\text{g}/\text{ml}$ was cytotoxic. Fig 2 illustrates that the initial burst of DNA synthetic activity routinely observed in these cultures on days 2–4, was followed by a relatively low level of DNA synthesis until days 8–10 when minoxidil-treated cultures showed a second burst of proliferation not seen in controls. This second peak was consistently higher than that seen on day 2. In experiments lasting up to 14 days, the second peak was again followed by a lower level of DNA synthesis. The magnitude of the newly synthesized peaks of DNA remained similar regardless of whether the specific activity of the [^3H]DNA was normalized to total DNA content or to protein concentration (Table I).

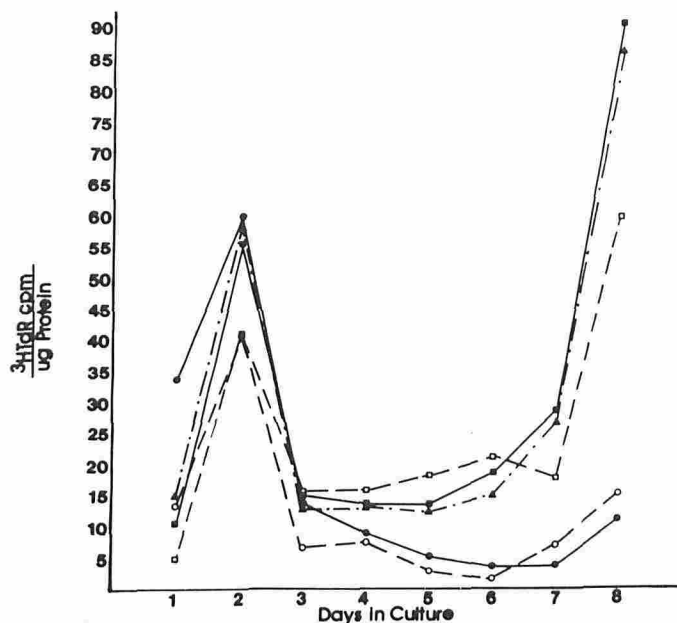


FIG 1. DNA synthesis in minoxidil-treated epidermal cell culture. Basal epidermal cells ($10^6/\text{ml}$) were plated with no additions (\bullet), vehicle (\circ), minoxidil 5 $\mu\text{g}/\text{ml}$ (\blacksquare), minoxidil 10 $\mu\text{g}/\text{ml}$ (\blacktriangle), and minoxidil 20 $\mu\text{g}/\text{ml}$ (\square). Cultures were pulsed daily with [^3H]dThd (1.25 $\mu\text{Ci}/\text{ml}$) for 4 h. Protein was measured as described by Lowry et al [6]. Data represent the mean of duplicate cultures. Variation was within 10% of the mean.

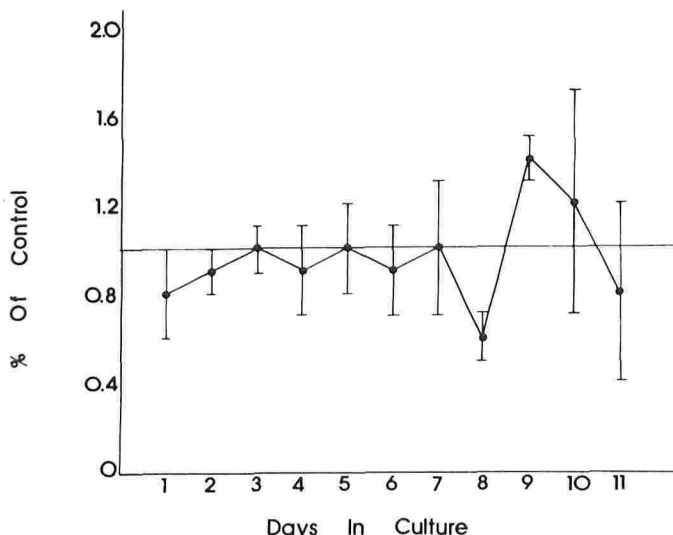


FIG 2. DNA synthesis in minoxidil-treated epidermal cell cultures expressed as percentage of control values. Data represent the mean (\pm SE) of 4 experiments each performed in duplicate as described in *Methods*.

Two morphologically different cell types could be distinguished in increasing numbers 4–5 days after minoxidil addition (Fig 3). Rectangular or fusiform cells with short processes, clearly defined cell borders, and centrally located nuclei were seen in clusters. In addition, some rectangular cells were multinucleated and contained multiple nucleoli. The second cell type was characterized by indistinct borders, and thinly spread cytoplasm. Many of these cells had long, thin interdigitating processes with nodelike thickenings. In general, their cytoplasm

TABLE I. [^3H]DNA in minoxidil-treated cultures normalized to total DNA or protein

Exp	2nd Peak DNA synthesis	% of Control	
		Normalized to DNA	Normalized to protein
1	Day 9	162	243
2	Day 9	149	168
3	Day 10	184	228

Each experiment represents the mean of duplicate points. Epidermal cultures were pulsed for 4 h with [^3H]dThd (1.25 $\mu\text{Ci}/\text{ml}$; sp act 20 mCi/mmol) and acid-precipitable [^3H]DNA was counted by scintillation. Assays for protein and total DNA content are described in [6,7].

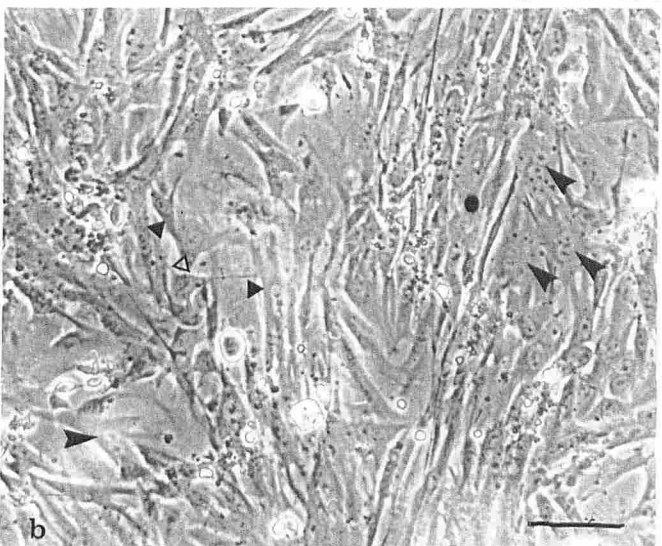
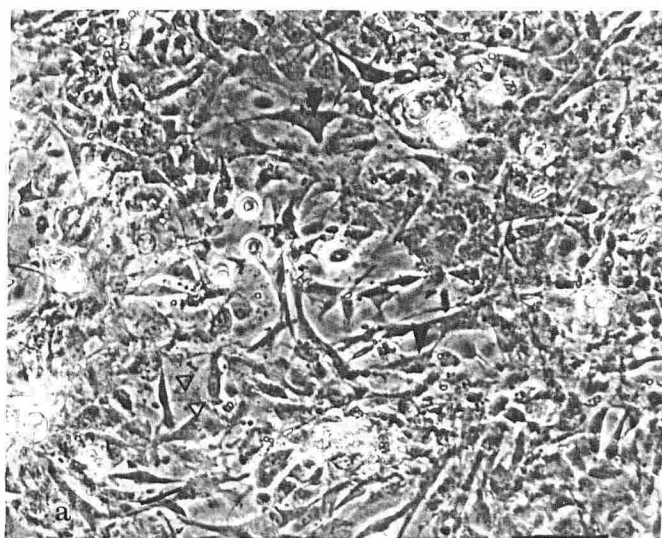


FIG 3. Epidermal cell cultures, 7 days (a), and 10 days (b) after addition of minoxidil. Rectangular cells with pseudopodia (\blacktriangleright) and vesicular cells (\triangleright) were prominent in cultures over 1 week of age. Multinucleated cells (\blacksquare) were frequently seen. Scale bar = 100 μm .

contained prominent vesicles and foamy inclusions (Figs 4, 5) which did not stain for lipid using the oil red O stain. Stratified areas composed of differentiating squames were present in all cultures. However, with time, the minoxidil-treated cultures developed numerous lacunae-like areas containing only rectangular and vesicular cells.

In order to demonstrate the epidermal nature of these two cell types, immunofluorescence microscopy with keratin-specific antibody was performed. Fig 6 shows that populations of cells with abnormal morphology stained positively for keratin, suggesting their epidermal origin. Preliminary studies assaying leucine aminopeptidase activity (as a marker of fibroblast contamination) and the percentage of cells having cornified envelopes (as a marker of differentiated epidermal cells) did not reveal differences between minoxidil-treated cultures and controls, suggesting that these differences do not arise from fibroblastic overgrowth.

Passaged Epidermal Cell Cultures

Subcultures of 5- to 6-day-old control and minoxidil-treated epidermal cells were initiated and maintained in minoxidil-free media. Untreated and solvent-treated cultures could not be passaged. In contrast, cells derived from minoxidil-treated primary cultures proliferated rapidly and could be repassaged

within 3–5 days. These cells maintained the altered morphology of the rectangular and vesicular cells (Fig 7). They grew haphazardly with much overlap and interdigitation of pseudopods. Cells in these cultures were identified as keratin-positive epidermal cells by indirect immunofluorescence microscopy utilizing antikeratin antibody (Fig 6c) although stains for keratin in formalin-fixed cultures were negative when assayed by less sensitive histologic methods.

Dermal Fibroblast Cultures

Preliminary studies of the effect of minoxidil on neonatal dermal fibroblasts were performed under the same conditions as those for epidermal cultures. Fibroblast cultures were approximately 80% positive for leucine aminopeptidase activity as detected by granular brown reaction products; this value is consistent with those reported in the literature [4].

Minoxidil-treated fibroblast cultures grew more rapidly than untreated fibroblasts and contained approximately 5% multinucleated cells. No fibroblasts stained for keratin when treated with the anti-keratin antibody.

DISCUSSION

These studies have demonstrated that minoxidil can have direct effects on the proliferation and morphology of epidermal cells in culture. The concentrations of minoxidil used in these experiments were comparable to those used clinically in the treatment of alopecia areata.

Minoxidil-enhanced DNA synthesis suggests that the drug can act as a mitogen for epidermal cells. The second burst of DNA synthesis, occurring at day 8, after cells have begun to differentiate, is temporally correlated with the appearance of increasing numbers of two morphologically different cell types. These rectangular and vesicular cells have an altered phenotypic appearance and do not stratify and die as do cells in control cultures. Unlike untreated cultures, which rarely survive past 21 days, minoxidil-treated primary cultures have survived over 60 days in culture.

Successful passaging of primary murine epidermal cultures is unusual. Consistent with the observations of others [14], we found in these experiments that although untreated cells may remain viable for periods of up to 2 weeks after passage, they do not proliferate but remain as small discrete foci of quiescent cells. In contrast, minoxidil-treated primary cultures could be passaged every 3–5 days and maintained in minoxidil-free media over a 2-week period. These cells retained the general features of rectangular shape or vesicular cytoplasm, were pleomorphic, and were multinucleated.

Phenytoin, a widely used antiseizure drug also has been associated with secondary effects of hypertrichosis [15]. The addition of phenytoin to epidermal cell cultures results in a similar pattern of enhanced DNA synthesis as observed in minoxidil-treated cultures (M.E.A.F. Alves, R.L. Cohen, and D.A. Chambers, manuscript in preparation). In addition, both drugs have also recently been associated with immunosuppressive activity [16–18].

The mechanism of action through which minoxidil is associated with hair growth is as yet unknown.

In clinical trials, topical application resulted in normal terminal hair regrowth at sites affected by severe alopecia areata. Histopathologic examination of posttreatment scalp biopsies revealed no change in the surface epithelium. However, there was normalization of hair follicle size and depth as well as a decreased perifollicular inflammatory cell infiltrate and a significant increase in the number of patent blood vessels [3]. One proposed mechanism of action for minoxidil-induced hypertrichosis is that of vascular dilatation [19,20] and increased cutaneous blood flow. However, the *in vitro* studies reported herein suggest that the drug could have direct effects on the epidermis at the site of application.

These *in vitro* studies show that minoxidil has direct mito-

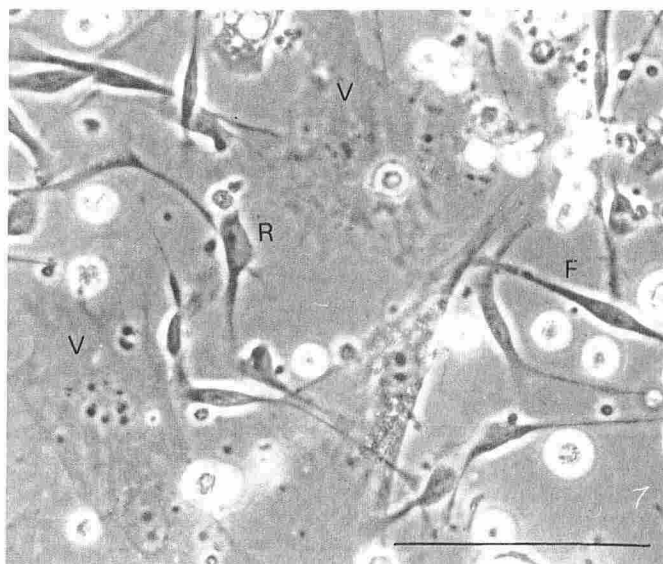


FIG 4. Fourteen-day minoxidil-treated culture. Rectangular (R), fusiform (F), and vesicular (V) cells are seen. Scale bar = 100 μ m.

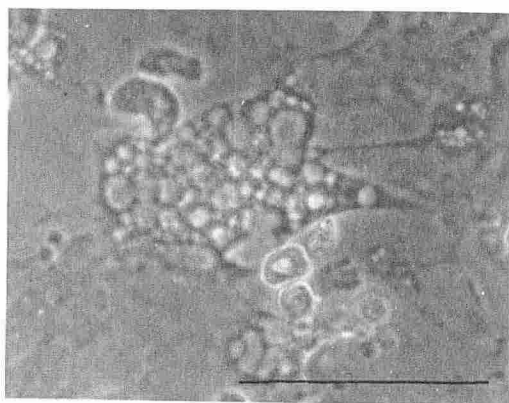


FIG 5. Vesicular cells in a 9-day minoxidil-treated culture. As the cultures aged, these cells enlarged, showing increased numbers of foamy inclusions and vesicles. Scale bar = 100 μ m.

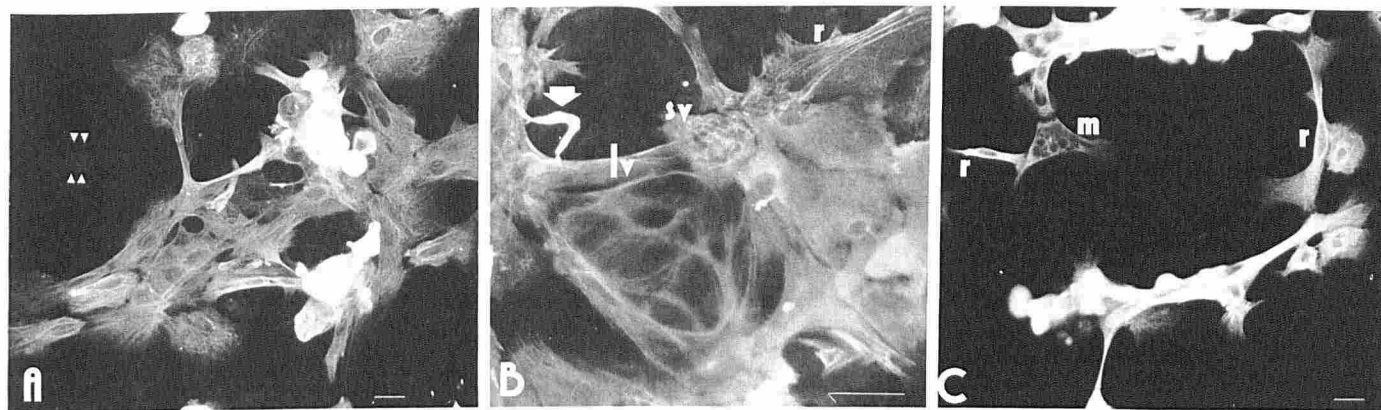


FIG 6. Detection of keratin by immunofluorescence microscopy. A, Minoxidil-treated 4-day primary epidermal cell culture. Note variation in intensity of staining. B, Minoxidil-treated 10-day primary epidermal cell culture illustrates keratin-positive large vesicular cell (*lv*), small vesicular cell (*sv*), a portion of a rectangular cell (*r*), and a fragment of a shed cornified cell (*arrow*). C, Cells from minoxidil-treated epidermal cell culture maintained in drug-free medium, 4 days after passage. Note rectangular (*r*) and multinuclear (*m*) cells. Minoxidil-treated fibroblast cultures show no fluorescence. Minoxidil-treated epidermal cell cultures reacted with preimmune serum show no fluorescence. Scale bars = 10 μ m.

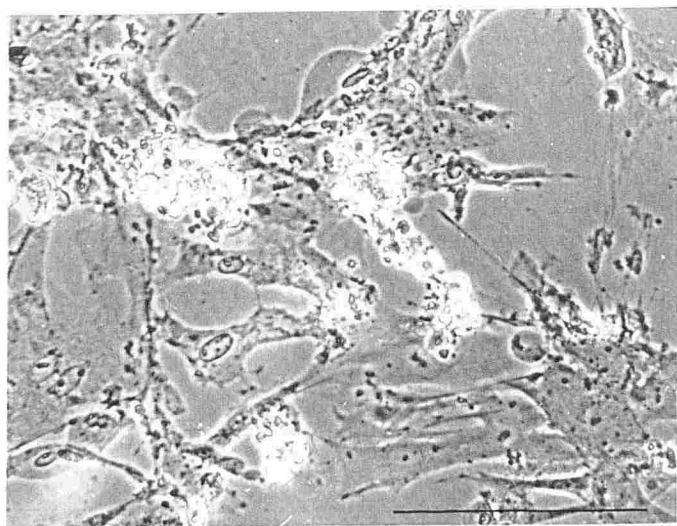


FIG 7. Subcultured cells derived from minoxidil-treated cultures. These cells, maintained in minoxidil-free medium, were multinuclear and pleomorphic. Scale bar = 100 μ m.

genic and morphologic effects on epithelial cells. Further use of in vitro epidermal cell culture systems may be valuable for dissection of the pharmacologic mechanisms through which hypertrichotic agents act. Additionally, since minoxidil alters epithelial cell proliferation and morphology in epidermal cell cultures, this drug may be a useful probe to examine regulatory mechanisms of epidermal homeostasis.

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